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Study of the Function of the Mold Specific Gene MS95 in DNA Repair in the Pathogenic, Dimorphic Fungus *Histoplasma capsulatum*

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Study of the Function of the Mold Specific Gene *MS95* in DNA Repair in the Pathogenic, Dimorphic Fungus *Histoplasma capsulatum*

by

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Abstract

Histoplasma capsulatum (Hc) is a dimorphic fungus that is the etiologic agent of the respiratory infection Histoplasmosis that is mediated by a shift from the mold phase to the pathogenic yeast phase. Genes have been identified that are specific to the mold or yeast phase in order to study the molecular biology of this shift. *MS95* was identified in a subtractive cDNA library that was enriched for mold-specific genes, and has been found to be homologous to the DNA damage-responsive gene *DDR48,* which functions in DNA repair in *Saccharomyces cerevisiae.* In order to elucidate the function of *MS95*, a loss of function mutant was created via allelic replacement. To determine if *MS95* is involved in DNA repair, wild-type and *MS95* knockout mutant strains were grown on *Histoplasma* macrophage media (HMM) supplemented with varying concentrations of 4-nitroquinoline 1-oxide (4-NQO) or paraquat dichloride. Growth was monitored for any changes between the two strains indicating that *MS95* plays a role in DNA repair. To confirm the results, the knockout mutant was complemented, and these complements were analyzed via real-time PCR. There was a significant difference in growth between the wild-type and knockout mutant strains when grown on plates that contained 40 and 60uM paraquat. There was not a significant difference observed between the two strains when grown on media containing 4-NQO. The results of the RT-PCR show that expression of *MS95* was restored, thus confirming successful complementation.

Key Words:
Histoplasma capsulatum
Dimorphic
MS95
DNA repair
4-Nitroquiniline-1-oxide (4-NQO)
Paraquat Dichoride

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Chapter 1: Problem Statement

Histoplasma capsulatum (*Hc*) is a dimorphic fungus that is the causative agent of the respiratory infection histoplasmosis. This disease is endemic to the Mississippi and Ohio River Valley flyways. New advances in molecular techniques have made it possible to study *Hc* on the transcriptional and translational levels. The lab in which the work for this project is being conducted has identified multiple genes that could be key role players in this vital shift. Because the yeast-phase of the organism is pathogenic, most labs study genes that are specific for this phase. This is useful in determining genes that would be good drug targets in the event of infection, but what if the shift, and thus progression of the disease could be stopped? Although the mold phase is not pathogenic, the main objective of this project is to determine the function of one of the few mold-phase specific genes that has been identified in *Hc*. The gene that is the focus of this project is the mold-specific gene identified *MS95*. This work on determining the function of *MS95* could be crucial to finding out how to stop the mold-to-yeast transition that leads to disease. Not a lot is known about its function since it has only been identified for a few years. Through NCBI GenBank and BLAST analysis, it is known, however, that *MS95* is homologous to the DNA damage responsive (*DDR48*) gene that functions in DNA repair in *Saccharomyces cerevisiae*. Because of this homology, the current hypothesis being investigated is that *MS95* plays a role in DNA repair in *Histoplasma capsulatum.* To determine if this is the function of the gene an *MS95*-expressing strain *Hc* WU27 as well as an *MS95* knockout mutant (USM14Δ*95*) created in our lab were subjected to a stress experiment in which the chemicals 4-Nitroquinoline N-oxide (4-NQO) and Paraquat Dichloride were used to create oxidative stress and ultimately DNA

damage. The strains were grown on the rich medium *Histoplasma* macrophage media (HMM) that had been supplemented with various concentrations of the chemicals, and growth was monitored for any phenotypic changes indicating recovery and the presence of DNA repair. To confirm that it is the expression of *MS95* causing the recovery in WU27, USM14Δ*95* was complemented with a functional copy of *MS95*. To do this, the *MS95* open reading frame was inserted into the expression vector pRPU1 and then transformed into the knockout via electroporation. The putative complement strains were analyzed via quantitative real time PCR to determine if complementation was successful in restoring relative expression of *MS95*. Future work includes subjecting the complement strains to the stress experiment, and it is hypothesized that the growth of these strains will be similar to that of the wild-type, further confirming the data from the initial stress experiment.

Chapter 2: Literature Review

Introduction to Fungi

Kingdom fungi are made up of organisms once thought to be plants. Deeper studies into the makeup of fungi showed that they are in fact quite different from plants. One example of these differences is how they acquire nutrients. Plants must use sunlight for photosynthesis to obtain nutrition, however, this is not the case with fungi. Plants are autotrophs, whereas fungi are heterotrophs, meaning they cannot make their own food. Some obtain nutrition through parasitism of other living organism, but most fungi are saprophytic heterotrophs, meaning they feed on dead or decaying matter. Because they do not need sunlight for growth, fungi can grow in a wider range of environments than plants. Another characteristic that separates them from plants is the way they reproduce.

Fungi can reproduce sexually or asexually. Asexual reproduction allows a large number of offspring without the need for mixing of DNA from two organisms, as is needed by plants that reproduce through pollination. Sexual reproduction occurs when two haploid gametes fuse together in a process called karyogamy. Asexual fungal reproduction occurs when two hyphae fuse together. Fungi can be heterothallic, meaning two complementary (often + and-) hyphae are fused together, or homothallic, in which the same mycelium can fuse to begin reproduction. Once hyphae are fused, gametangia are produced which function to produce new spores. Fungi are classified into the following three categories based on the method and structures they use for spore production: ascomycota, basidiomycota, and zygomycota.

Ascomycota is the largest phylum of fungi, containing 64,000 identified species. They are present in almost every environment, and are one of the most diverse eukaryotic phyla. Members of the phylum ascomycota are often hard to phylogenetically map because most of them are only asexual (Schoch et al., 2009). Ascomycetes are defined by their use of asci to house spores in reproduction, with a few exceptions. Ascomycetes are beneficial to humans in many ways. Without these fungi, humans wouldn't have many types of cheese, alcoholic beverages, or antibiotics. Penicillin, the first antibiotic ever used, was synthesized after Alexander Fleming discovered an anti-bacterial property in *Penicillium chrysogenum*. The fermentation process used by *Saccharomyces cerevisiae* is necessary for the production of beer, and cheeses such as bleu cheese get their tangy flavor from fungi. While there are many beneficial ascomycetes out there, many others are among the fungal pathogens of the world. *Candida albicans* is a well-known pathogenic ascomycete that causes oral and genital infections commonly known

as thrush; the condition commonly known as "athletes foot" is caused by an ascomycete in the genus *Trychopyton*. The pathogenic ascomycete that is the focus of this project is the dimorphic *Histoplasma capsulatum*, that is the etiological agent of the respiratory infection histoplasmosis.

Histoplasma capsulatum (*Hc*) is a heterothallic ascomycete that is endemic to the Ohio and Mississippi River Valley flyways in the United States. It is one of three infectious variants in the genus *Histoplasma*. *Histoplasma duboisii* and *Histoplasma farciminosum* are two other pathogenic species. *H. duboisii* is primarily found Africa while *H. farciminosum* mostly infects horses and mules (Retallack and Woods, 1999).

Classification of *Histoplasma* Strains

Histoplasma capsulatum has been classified in many ways since it was discovered by Samuel Darling in 1905. One of the first characteristics used to classify *Hc* was the polysaccharide content, namely α 1-3-glucan, of the cell wall. Those that were classified as type I, such as the G217B strain, have negligible amounts of the polysaccharide, whereas type II, such as the G186A strain, have an abundance of α 1-3-glucan in their cell walls (Domer, 1971). Early studies did not link polysaccharide content with virulence. Later studies, however, showed a correlation between virulence and cell wall makeup. It was proposed that α 1-3-glucan could be linked to virulence after observing lack of disease in mice infected with type I strains (Klimpel and Goldman , 1988). A study using RNA interference formally proved that α 1-3-glucan acts as a virulence factor (Rappleye et al., 2004). It was thought that the α 1-3-glucan acted as a protective coating for the cells once inside the phagolysosome because it could keep the immune system from recognizing the β glucan on the inner membrane of the cells. Another strain of *Hc*,

the Downs, strain was later identified. It was classified into its own group because, although it is avirulent in mice, its avirulence is caused by a factor other than glucan content. At physiological temperature, Downs is temperature sensitive. Its highest heat shock response occurs at 34°C, rendering it nonpathogenic (Maresca and Kobayashi, 1989).

Dimorphism

What separates *Hc* from most commonly studied fungi, pathogenic or not, is the fact that it is dimorphic. This ability to exist in two forms exhibits *Hc*'s highly evolved capability of adapting to its environment. Because of this, the mold and yeast phases have different nutrition requirements, likely based on what is available in the different environments. In the environment, *Hc* exists as a multicellular differentiated mold (which can be replicated in the lab by incubation at 25°C). It is typically found in soil that has been contaminated with bird and/or bat guano. In the mycelial phase, *Hc* reproduces heterothallically with + and – mating type hyphae (Retallack and Woods, 1999). It has very simple nutritional needs, utilizing glucose and ammonia for Carbon and Nitrogen, respectively. The hyphae are 1.25 to 2 μm in diameter with walls that are around 20 nm thick. Once certain conditions are met, *Hc* can undergo a shift from this multicellular mold phase to a unicellular yeast phase. Once mold cells are exposed to the 37°C environment, they begin shifting to the yeast form. In order to maintain the yeast form, *Hc* requires sulfhydryl groups such as cysteine or cystine for redox potential. It also needs biotin, thiotic acid and/or thiamine, in addition to glucose and nitrogen sources. Cell walls of the yeast phase of *Hc* are thinner than those of the mold phase. Reproduction is also much different in yeast than in mold—progeny yeast are produced

by budding from the innermost cell wall layer of the parental cell (Maresca and Kobayashi, 1989).

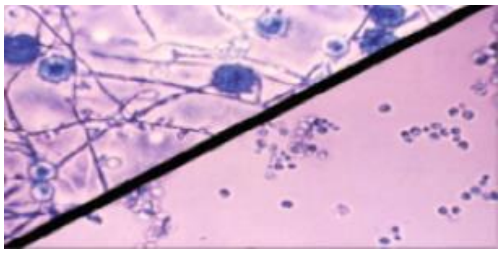

Figure 1. *Histoplasma* mold (as depicted in the upper left)and yeast (as depicted in the lower right) stained with lactophenol blue.

Factors Involved in Dimorphism

One of the main instigators of this shift in *Hc* is temperature. Because of this, *Histoplasma* can be manipulated into shifting in the laboratory by using incubators that are kept at 25° and 37°C (Maresca and Kobyashi, 1989). This necessity for temperature change is evident in the fact that the two phases usually exist at drastically different temperatures (Eissenberg and Goldman, 1991). Without shifting the temperature, yeast will stay as yeast, or mold will stay as mold.

Another important factor in shifting from mold to yeast upon inhalation is oxidation-reduction potential (Maresca et al., 1978). Without nutrients such as cysteine or cystine, mold cells cannot shift to the infectious yeast phase. This nutrient is only needed in the yeast phase. Its absence does not affect mold cells or the shift from yeast to mold. In a study performed by Medoff et al 1897, a sulfhydryl-blocking agent p-chloromercuriphenylsulfonic acid (PCMS) was added to media in which mold cells were growing, and when the cultures were shifted to 37°C, no yeast grew. Even after many

passages in media that did not contain any PCMS, the mycelia never shifted to yeast. The mold still grew, which meant the yeast form was not necessary for survival in warmer temperatures. When yeast cells were grown in the media and then switched to 25°C, there normal mold grew, but these cells could not switch back to yeast once put back in the 37°C environment. The results of this experiment showed that cysteine is necessary for the shift from infectious mold to pathogenic yeast. To further study the effects of the lack of cysteine on the organism, mold grown in the presence of PCMS was injected into mice, and tissue was examined after a few days. The mice were healthy and there was no evidence of *Hc* in their systems, illustrating that this dimorphic shift is vital to the pathogenicity of *Histoplasma capsulatum* (Medoff et al., 1987).

Introduction to Histoplasmosis and the Disease Process

Histoplasmosis was first observed by Dr. Samuel Darling in 1905. While performing an autopsy on a patient in Panama, he noticed granulomas in the lungs and took samples to culture. The samples contained small endothelial cells that he thought were encapsulated protists. Because he isolated the cells from histiocytes (macrophages), Darling [incorrectly] named the organism *Histoplasma capsulatum* (Bradsher 1996). Two of the patients had died from recurring cases of pneumonia, while the third had died as a direct result of the disease.

Histopasmosis is the result of inhaling aerosolized spores from contaminated soil. *Hc* is common in areas where there are a lot of birds or bats. A study of bat caves in Florida was conducted after a college student developed histoplasmosis following a class trip to explore a cave, and it proposed a direct correlation between exposure to contaminated soil and increased risk of the disease (DiSalvo et al., 1970). There was also

evidence of bird and bat association with the disease when an attic of a family who had all succumbed to the disease was examined after they moved out of their house (Emmons, 1958). While the risk of infection is high when simply being exposed to the soil, the risk rises exponentially if there are factors leading to a large disruption of soil in an infected area, such as bulldozing on a construction site (Chick et al., 1981). Once spores are aerosolized, they can be inhaled and enter the host's lungs. If the host's immune system does not begin to clear the pathogen, histoplasmosis can develop.

Once in the warmer environment inside the host, the heat shock response is initiated, and the dimorphic shift begins. The yeast cells are then engulfed by macrophages, where they will reside and travel throughout the body, essentially using the macrophages as a shield from the rest of the immune system. This is no small feat in itself, though. Because of its job, the environment inside the macrophage is quite harsh. In order to survive once engulfed, *Hc* must find a way to get certain nutrients and adapt (Eissenberg et al., 1993). One way of doing this is to modulate the pH of the acidic phagolysosome.

Inside the macrophage is the phagolysosome, which contains degradative enzymes that function best at low pH. This is how most intracellular pathogens are destroyed. *Hc* is one of the only pathogens identified that can raise the pH of the phagolysosome to near neutral. This mechanism is not completely understood, but it has been shown that this failure of the phagolysosomes to acidify is not the result of fusion inhibition, as is the case with other pathogens that can survive macrophage engulfment (Eissenberg et al., 1993).

Along with neutral pH, *Hc* must have a way to acquire iron and calcium to survive. Many hosts will respond to infection by sequestering nutrients the pathogen needs (Retallack and Woods, 1999). This is true of iron and calcium inside macrophages. Because of this, *Hc* has special mechanisms it uses to acquire these nutrients. Calcium is needed for both the mold, and yeast phases, but it is easier to come by in the soil than in the macrophage. To overcome this, *Histoplasma* has developed a protein to "grab" any bit of calcium it finds when in the yeast phase. This protein is the calcium binding protein (CBP), and it is observed only in the yeast phase (Batanghari and Goldman, 1997). Along with calcium, *Hc* needs iron to survive. Since iron is a key component of red blood cells in humans, there is very little free iron. *Histoplasma* uses a receptor called a siderophore to bind iron. It also uses the enzyme ferric reductase that allows the organism to acquire iron from siderophores, inorganic salts, or iron-binding host proteins such as hemoglobin.

Diagnosis and Treatment

Hundreds of thousands of cases of histoplasmosis are diagnosed in the United States every year (Davies et al., 1978). The majority of these cases are in the endemic Mississippi and Ohio River Valley areas, although, with the high mobility of the population, more infections are occurring in areas where *Hc* is not commonly found. Of these half a million cases of histoplasmosis, the vast majority are asymptomatic. Most experience mild symptoms that mimic a cold or a mild case of influenza. Those that do have symptoms usually experience fever, weight loss, shortness of breath, and coughing, symptoms common to most respiratory illnesses (Wheat, 1994). While healthy people may experience mild to severe cases of histoplasmosis, those most susceptible to

disseminated and fatal histoplasmosis are immunocompromised patients such as those with HIV/AIDS, chemotherapy patients, or those taking immunosuppressants for organ transplants or autoimmune diseases (Wheat et al., 1982). Once the disease has disseminated throughout the body, it can lead to multiple organ failure, which is fatal.

There are a number of methods used to diagnosis histoplasmosis. One of the earlier tests involved culturing a sample of sputum, blood, or tissue from infected patients. This method was accurate in diagnosing a high percentage of chronic and disseminated cases. Another common diagnostic test used is a skin test. A small amount of the histoplasmin antigen is injected into the skin, and the spot is observed after a couple of days. A visible reaction at the injection spot is indicative of *Hc* exposure, because it means that the patient's body has made antibodies to *Histoplasma*, which only occurs after exposure. This is only effective in diagnosing people four or more weeks after exposure, as that is the average amount of time the body needs to produce antibodies to a pathogen. This method is not effective in diagnosing those with HIV/AIDS because their immune system no longer functions to properly produce antibodies (Diamond, 1991). Along with early cases of histoplasmosis, this method is not as effective in diagnosing immunocompromised patients. The most accurate method used to diagnosis histoplasmosis is a chest x ray. An x-ray of someone who has the disease will show calcifications in the lungs.

Various antifungal drugs are available to treat histoplasmosis. While most are effective at clearing the infection, many have detrimental side effects, as well. One of the most common and effective drugs used to treat histoplasmosis is Amphotericin B. It is highly effective in clearing chronic (81%) and disseminated (68%) infections. Because it

has been known to cause renal failure and other detrimental side effects, the toxicity of Amphotericin B discourages its use over an extended period of time. Once the infection is initially controlled with Amphotericin B, other medications such as Itraconazole, Ketoconzole, or Fluconozole are usually administered (Wheat, 1994). These drugs are often less effective than Amphotericin B at treating the disease (Graybill, 1995). While these are also successful at treating the disease, they can also cause severe side effects, or have adverse effects on other medications being used at the same time (Wheat, 1994). This lack of effective treatment is the driving force for much of the research being conducted today. In order to develop treatments with fewer adverse side effects, the molecular biology of *Hc* must be understood in more depth.

Relevance of My Project

While much of the biology of *Hc*, including protein expression and environment adaptation, has been studied, a lot of current research focuses on the genetics. This is important, because there must be gene regulation involved in the shift from mold to yeast, vice versa. Finding new treatments for histoplasmosis is dependent on identifying factors involved in the shift (Maresca and Kobayashi, 1989). If genes essential to dimorphism can be isolated, it is possible that new drugs could be developed that could target the protein products of said genes, shutting off the dimorphic shift that leads to disease. Up- or down-regulation of certain phase-specific genes in *Hc* could be one of the main players in maintaining dimorphism in the organism (Tian and Shearer, 2001). A lot of the current work on *Hc* is being done to identify these genes involved in dimorphism. Differential gene expression has been confirmed in *Hc*, and there have been some phase specific genes identified, most of which are yeast-phase specific, such as the gene YPS3

(Hwang et al., 2002). Yeast-phase genes are often studied in depth because the yeast phase is associated with the disease. YSP3 has been studied to some extent because its protein induces toll-like receptor signaling in the host (Aravalli et al., 2008). While some of the yeast-specific genes are important for pathogenicity, none have been found to be necessary for the dimorphic shift observed in *Hc* (Retallack and Woods, 1999). Because yeast-phase genes have been studied in so much depth, not much has been done to identify and characterize mold-specific genes in *Hc*. Because of this, a large piece of evidence to how the shift occurs could be missing. A few mold-specific genes, such as *M46* and *MS8* have been identified. It is possible that the down-regulation of genes such as these or others could be necessary for initiating the shift to the yeast phase (Tian and Shearer, 2001). This project focuses on a particular mold-specific gene, *MS95*. The purpose of this study is to characterize *MS95* to determine its role in *Hc*. The two functions that will be examined are involvement in dimorphism and DNA repair. This project is a part of the only work being done to determine the function of this gene. It is also being conducted in one of the only labs that even study mold-specific genes, making it relevant to the work being done to find more efficient methods of treating histoplasmosis.

Because not much is known about the mold-specific genes, especially the gene involved in this project, it is necessary to look at homologs to get an idea of the possible function of *MS95*. An organism that is closely related to *Hc* is *Saccharomyces cerevisiae*. *MS95* has been discovered to be homologous to the *S. cerevisiae DDR48* gene which is known to play a role in repairing DNA damage. This gene is up-regulated in the organism in response to experiencing DNA damage. It is up-regulated most when *S.*

cerevisiae is exposed to heat or mutagenizing chemicals such as 4-NQO. The gene consists of two overlapping reading frames that code for a 45kD protein, one of which is actually expressed. Upon experiencing stress, three transcriptional start sites are utilized, as well. Although its function is not directly understood, the protein for which *DDR48* codes, *DDR48p*, is essential to its function in DNA repair (Sheng and Schuster, 1992). A study conducted by McLanahan and McEntee found that *DDR48* is dually regulated by heat shock and DNA damage in *S. cerevisiae*. This study also showed that the transcription start sites were used in equal frequency in both stress situations.

It is this homology to *DDR48* that led us to our hypothesis that *MS95* is involved in DNA repair in *Histoplasma.* To determine if this is, in fact, its function, WU27 as well as USM14Δ*95* strains were grown on solid HMM that contained various concentrations of the DNA-damaging chemicals 4-Nitroquinoline N-Oxide or Paraquat Dichloride, and the growth was monitored for any phenotypic changes between the two strains. To confirm the results of this experiment, USM14Δ*95* was complemented via electroporation of the vector pEMS01 that contained the *MS95* open reading frame. Complementation was confirmed via quantitative real-time PCR. Future work includes subjecting the complements (USM11, USM12, and USM13) to the stress experiment.

Chapter 3: Materials and Methods

Strains and Growth Conditions

The strains that were used in this project were the WU27, a G186AS Δ*ura5-11*, background strain from Washington University, and *MS95* knockout mutant strain (USM14Δ*95*). Cells were maintained in *Histoplasma* macrophage media (HMM) which contains per liter 18.2 g glucose, 10.7 g Sigma F12 HAM, 1.0 g glutamic acid, 84 mg L-

cysteine, and 5.96 g HEPES sodium salt (Worsham and Goldman, 1988). Cultures were supplemented with 1 mg/mL uracil when strains auxotrophic for uracil were used. The media was titrated to pH 7.5 and filter sterilized. WU27 and USM14Δ*95* were grown in HMM with uracil. Yeast was maintained at 37°C with shaking at 200 rpm, and 500 µL of cells were passed to 50 mL of HMM every four days. Mold cells were maintained at 25°C with shaking at 200 rpm, and 1 mL of cells were passed to 50 mL of HMM every seven to ten days.

Stress Experiment

Solid HMM with uracil was prepared as described above. A 0.1 mg/mL stock of 4-Nitroquinoline N-oxide (Sigma Aldrich) was prepared in 100% acetone and supplemented at the following concentrations: 1 ng/mL, 2.5 ng/mL, 5 ng/mL, and 10 ng/mL, respectively. A 2 mM stock of Paraquat Dichloride (Fluka) was prepared in Millipore water and supplemented at the following concentrations: 20 µM, 40 µM, and 60 µM, respectively. WU27 and USM14Δ*95* were grown to mid log (approximately 200-300 reading with a Klett-Summerson colormeter) at 37°C. A 1:100 dilution was prepared and then quantified with a hemacytometer. Once quantified, the cells were diluted with pre-warmed HMM to reach a final concentration of $1x10^4$ cells/µL Both strains were spotted in triplicate in 5 µL volumes on 100x100 mm square plates to yield a final concentration of $5x10^4$ cells/spot. They were allowed to dry and then incubated at 25°C to stimulate mold-phase growth. Plates were incubated for five days to allow the spots to grow and become visible. Starting at day 6, the plates were imaged with a Dino-Lite digital microscope every 24 hours through day 10. The diameter of each spot was

measured for images taken on day 8 using the Dino-Lite software. This experiment was repeated in biological triplicates.

RNA Extraction

The mold phase was harvested by vacuum filtration onto a Whatman, Grade No. 1, 11 μm filter paper. RNA was extracted by glass bead disruption in acidic phenol as follows: 45 mL of culture was harvested and added to a 1.5 mL screw-top micro-centrifuge tube containing 400 μL of 5:1 acidic phenol/chloroform, 400 μL of extraction buffer (0.1M Na acetate, 0.2 M NaCl (pH 5.0), and 0.2% SDS), and 300 μL of 0.5 mm acid-washed glass beads. Cells were disrupted via shaking on an MP Fast Prep – 24 vortexer 4.0 m/s in 30 second intervals with a 1 minute intervals on ice for a total of 2 minutes of vortexing. Tubes were centrifuged at 12,000 X *g* for five minutes at 4°C to pellet the cell debris. The aqueous layer was transferred to a 1.5 mL eppendorf tube containing 1 mL of cold 100% ethanol and mixed gently. Tubes were then stored at -80°C overnight to maximize the RNA yield.

Quantitative Real-Time PCR Analysis

RNA from wild-type, USM14Δ*95*, USM11, USM12, and USM13 mold samples was extracted as described above, and cleaned up using an Aurum™ Total RNA minikit (#732-6820) per the manufacturer's protocol. 4 μg of RNA was reverse-transcribed using a Maxima First Strand cDNA Synthesis Kit for RT-qPCR (#K164). The reaction mixes were prepared using a SYBR Green/ROX qPCR 2X Mastermix (Maxima) mixed with 500 ng of cDNA for each sample and 0.5 μM of the specific primer for each gene according to manufacturer's protocol. Analysis was performed using a BioRad CFX96 Real-Time System using the following protocol: 95°C for 10 minutes followed by 40

cycles of 95°C for 15 seconds, 53°C for 30 seconds, and 72°C for 30 seconds. Relative expression levels were normalized to *GAPDH* transcript and then analyzed using the ΔΔCt method.

Construction of *MS95* Complement

The expression vector pRPU1 containing the *MS95* open reading frame was linearized with PacI (New England Biolabs) in order to expose the telomeric repeats. The desired fragment was separated from the cut tet marker via electrophoresis on a 0.7% agarose gel that had been stained with 10 mg/mL ethidium bromide and visualized with a UV light box (420 nm). The ~8kb fragment was recovered using an Epigenetics Zymoclean Gel Recovery kit according to manufacturer's protocol. The linearized vector was then transformed into USM14Δ*95* yeast cells via electroporation using an Invitrogen Electroporator at the following settings: 750 V, 71 μF, and 150 Ω (which gives a pulse of 3,750V/cm for 10.6 ms). The cells were plated on solid HMM without uracil and incubated at 37°C for 10-14 days to allow colony growth. Once colonies were visible, 10 were picked and streaked out onto solid HMM ura(-) and incubated at 37°C for 10 days for screening. Once streaks had grown, they were inoculated into 5 mL of liquid HMM ura(-) and incubated on a shaking roller drum for 5 days at 25°C. RNA was extracted as described above and expression was quantified via real-time PCR as described above.

Chapter 4: Results

Figure 2 shows the proposed sequence of *MS95*. According to the Broad Institute, *MS95* includes 1499 nucleotides and 3 introns, and it codes for a protein that contains 316 amino acids.

Figure 2. Sequence of *MS95* (http://broadinstitute.org/).
The translated sequence of 316aa is shown on top, and the 1499 nucleotide coding region is shown on bottom.

The goal of this project was to determine if the mold-specific gene *MS95* plays a role in DNA repair in *Hc*. For the stress experiment, WU27 and USM14Δ95 were spotted in triplicate on solid HMM with uracil that had been supplemented with varying concentrations of either 4-NQO or paraquat. They were grown at 25°C for 5 days to allow visible mold growth, and then analyzed with a Dino-Lite digital microscope. Results are shown for measurements taken at Day 8 after initial spotting.

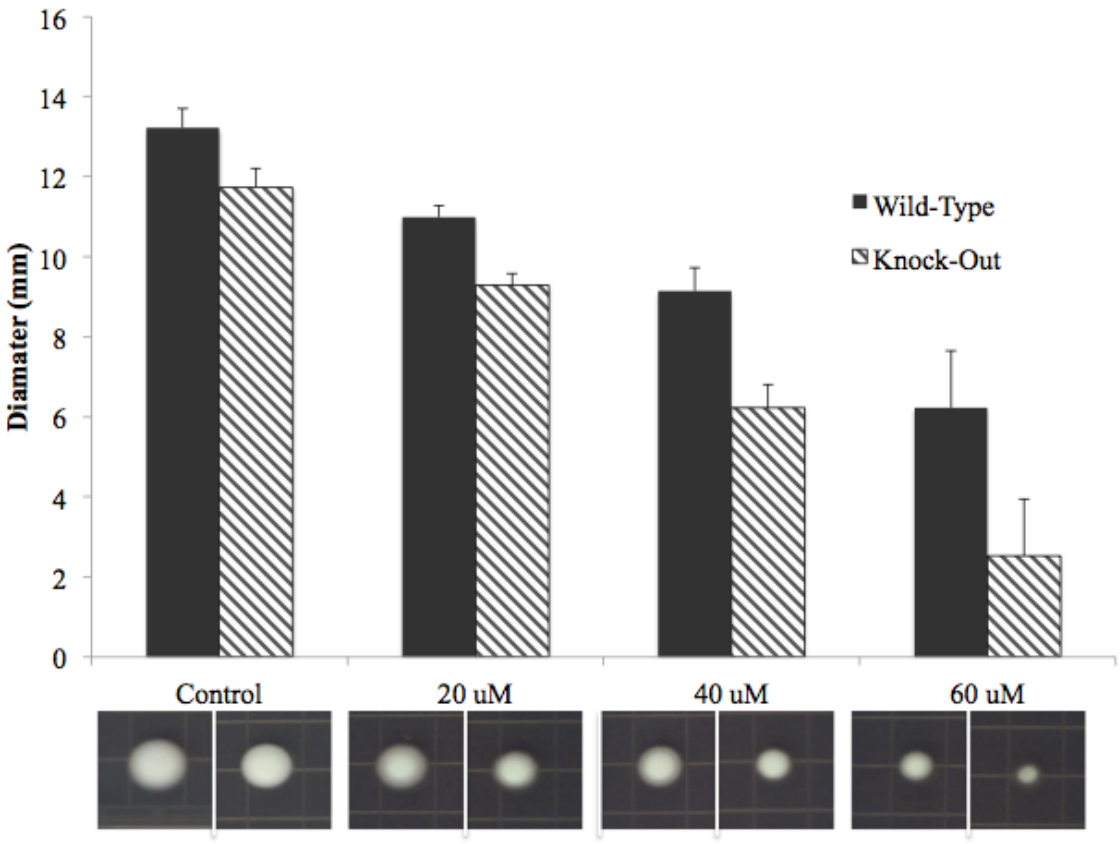

Figure 3. Stress Experiment Results for Growth on Paraquat.
The top represents diameter of wild-type (solid column) and and knock-out (dashed column). The bottom is a visual representation of the data.

As shown in Figure 3, there appeared to be a difference in growth between WU27 and USM14Δ*95* when grown on media containing paraquat. While there is not a statistically significant difference in growth on the plates that were supplemented with 20uM, there is a noticeable difference between strains on the higher concentrations. When grown on 40uM and 60uM paraquat, the USM14Δ*95* strain shows significantly smaller diameters than that of WU27.

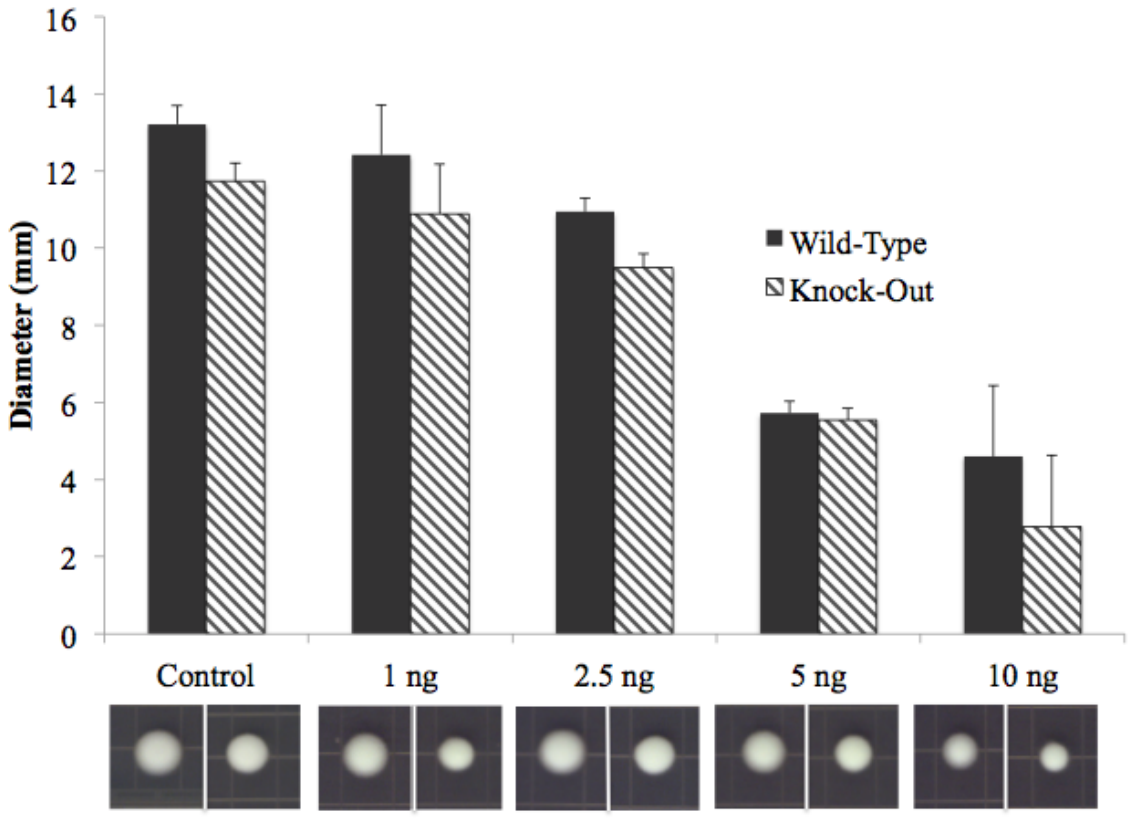

Figure 4. Stress Experiment Results for Growth on 4-NQO.
The top represents diameter of wild-type (solid column) and and knock-out (dashed column). The bottom is a visual representation of the data.

Growth on plates supplemented with 4-NQO is shown in Figure 4. This growth is different from that on paraquat in that there is not really a statistical difference in growth between strains for any of the concentrations. While there is an increase in inhibition of growth seen as the concentration increases, this inhibition appears to be affecting both strains equally.

To confirm that the results seen with growth on paraquat is due to the absence of *MS95* in the knockout mutant, we created a complement strain by inserting the open reading frame of the gene into a telomeric vector and then transforming it into USM14 via electroporation.

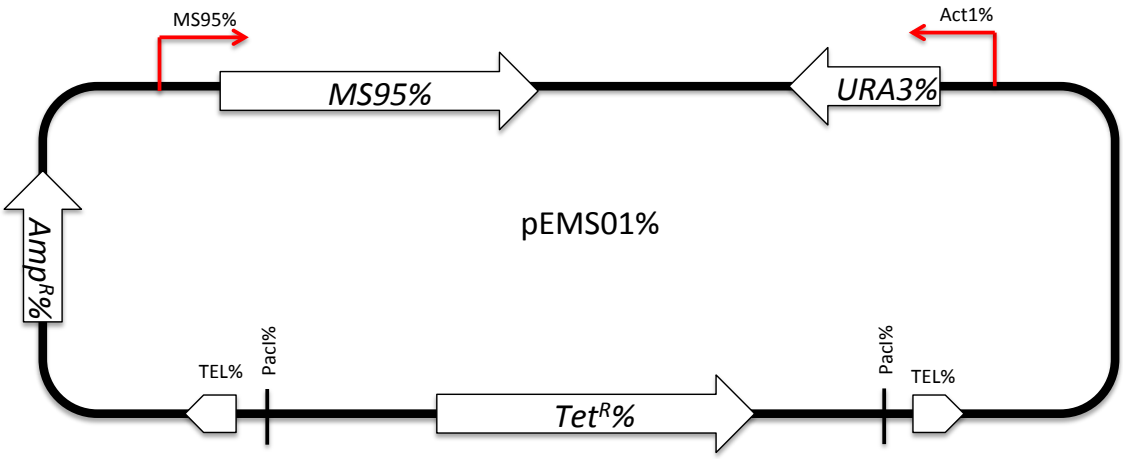

Figure 5. Plasmid Map for *MS95* Complement (pEMS01).
URA3 encodes orotidine 5-phosphate decarboxylase which is used as a positive selector for transformants. *Tet*^{R} encodes resistance to the antibiotic Tetracycline. TEL represents telpomeric repeats in the vector. PacI represents the restriction site used for exposing telomeric repeats. *Amp*^{R} encodes for resistance to the antibiotic Ampicillin. *MS95* represents the open reading frame for the gene.

In order to expose the telomeric repeats on the complement vector, the tet marker had to be cut out via restriction digest before electroporation into USM14Δ*95* (Fig. 5). PacI was used for this digestion, and the reaction was then separated via denaturing agarose gel electrophoresis (Fig. 6).

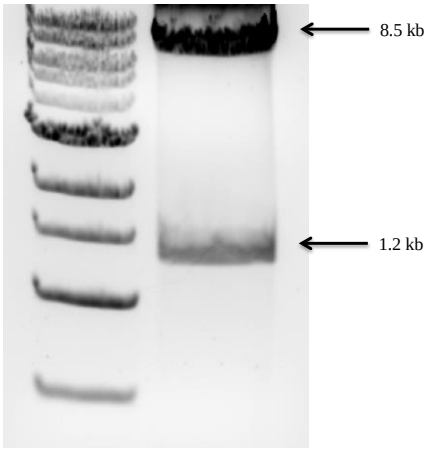

Figure 6. Restriction Digest of pEMS01 with PacI.
The 1.2 kb fragment contains the tet marker that was cut out to expose the telomeric fagments. The 8.5 kb fragment contains the open reading frame of *MS95* that was transformed into USM14Δ*95*.

The image of the gel shows proper cutting by PacI. The 1.5 kb band represents the tet marker, while the 8.0 kb band represents the *MS95* reading frame attached to the pRPU1 vector. This larger band was recovered and electroporated into USM14Δ*95*. Once colonies were selected and grown as mold, RNA was extracted for quantitative real-time PCR to analyze relative expression of putative complement strains.

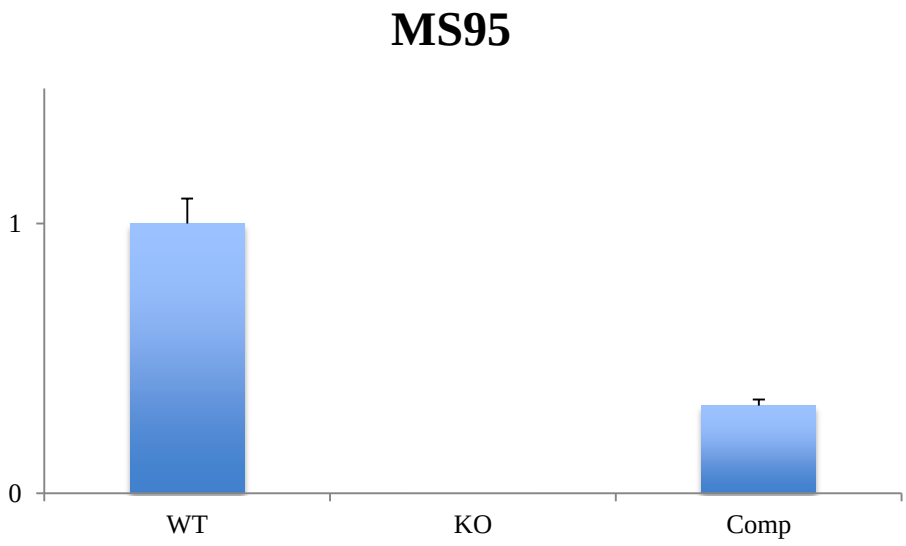

Figure 7. Quantitative Real-Time PCR for Relative Expression of *MS95*. Depicted above in the far left is the wild-type (WT), in the center is knock-out (KO), and in the far right is the *MS95* complement (Comp).

As seen in Figure 7, when normalized to *GAPDH* expression, it can be seen that there is a restoration of wild-type expression. These results indicate that while complementation was not 100% efficient, restoration of expression was still observed.

Now that USM14Δ*95* has been successfully complemented, future work includes subjecting these complements to the same stress experiment. It is hypothesized that if the lack of *MS95* expression is leading to the decreased growth in USM14Δ*95*, the complemented strain should see a recovery of growth and spot diameters that are similar to that seen in WU27. In order to further examine the recovery of the wild-type strain on

paraquat, production of the anti-oxidant supeoxide dismutase will be analyzed in both WU27 and USM14Δ*95* via quantitative real-time PCR. Because of the nature of the damage caused by paraquat, it is hypothesized that USM14 will show decreased, if complete absence, of superoxide dismutase if the gene is involved in DNA repair that was the result of oxidative stress.

Chapter 5: Discussion

As seen in Figure 3, there is a difference in growth observed between WU27 and USM14Δ*95* when grown on HMM that was supplemented with paraquat. It is not surprising to see this difference in diameter increase as the concentration of the chemical in the media increased. Although there is some observable inhibition of growth of WU27, that of USM14Δ*95* is much greater, suggesting less repair of the DNA. Because of this, it is reasonable to suggest that there is some factor leading to the larger diamater seen in the wild-type that is absent in the knockout. Because of the nature of these chemicals, this damage is most likely inflicted on the DNA. When the DNA of an organism is damaged, reproduction and growth are challenged. The damage seen in strains grown on media supplemented with 4-NQO are somewhat different than those observed for paraquat. As the concentration of 4-NQO increased in the media, the inhibition of the growth did as well. What is different about this chemical is that this inhibition of growth was fairly similar between both strains. There was not a big difference in diameter between WU27 and USM14Δ*95*; the growth declined in both strains.

While these results may appear at first to refute the original hypothesis that *MS95* is involved in DNA repair in *Hc*, it does not if the hypothesis is revised to take into account the different types caused by the two chemicals. While they both cause oxidative

stress in the cell, the type of DNA damage they cause it quite different. Paraquat causes more indirect effects on the DNA through oxidative stress that is mediated by the production of toxic free radicals, whereas 4-NQO more directly damages the DNA, which could also explain the damage it caused to both strains. In the cell, paraquat is reduced via redox cycling where it then reacts with molecular oxygen to produce free radicals that are toxic to the cell. These free radicals are typically combatted with compounds or enzymes that have anti-oxidant properties, the most common one being superoxide dismutase (Bus and Gibson, 1984). The damage caused by 4-NQO mimics that that of UV damage by directly damaging the strands of DNA. This chemical binds to DNA to make harmful Adenine and Guanine adducts, which must be repaired via excision repair (Kondo, 1977). Because of these vastly different pathways, it can further be hypothesized that *MS95* is more likely involved in the repair of stress caused by free radicals than that caused by direct DNA binding. This can be further supported by a study conducted in our lab in which UV was used to stress wild-type and knockout mutant strains. The results observed were highly similar to the results we obtained with 4-NQO.

By looking at the map of the complement construct and the gel electrophoresis image, it can be seen that the correct fragment of DNA was recovered and electroporated into USM14 to create the putative complement strains. The results of the real-time PCR show that complementation was successful in three putative complements. Although it is not 100%, expression of *MS95* was still restored. It is highly uncommon to achieve 100% complementation when inserting a knocked out gene into a vector that must be maintained by the cell. This also confirms that *MS95* expression seen in the complement

strains is the result of the result of the vector, and not the native genome of the strain. From this it can be concluded that relative expression of *MS95* in USM11, USM12, and USM13 is the result of complementation, not unsuccessful knockout. Based on the results of this project, it can be concluded that *MS95* is playing some role in DNA repair in *Histoplasma capsulatum*. While the exact role is still unknown, this data provides groundwork that gives direction for future work with this gene.

Future Directions

To confirm the results seen with paraquat-supplemented media, USM11, USM12, and USM13 will be subjected to the stress experiment described above. This will be done in triplicate, as well. Although complementation was not 100% efficient, we are hypothesizing that the diameter of the spots for USM11, USM12, and USM13 will be similar to those of WU27 when grown on media supplemented with paraquat. Because 4-NQO appears to be so toxic to the cells, it is expected that the complement strains will show similar inhibition, as well.

To determine if *MS95* has a role in repairing oxidative stress, real-time PCR will be conducted to analyze the expression of the *Superoxide Dismutase 3* (*SOD3*) gene. If involved in the production of antioxidants, we are expecting to see a decreased amount of relative expression of *SOD3* in USM14Δ*95* when compared to that of WU27. The results of these two future experiments could give a somewhat definitive function to *MS95*.

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Appendix A: Primers Used for Quantitative Real-Time PCR

MS95 forward: 5’ AGT GGC AAC GAC AGC TAT 3’

MS95 reverse: 5’ GAA TCA CTC CCT CTT CCA GTA TTT 3’

GAPDH forward: 5’GAA ACC AGT CAC CTA CGA TCA 3’

GAPDH reverse: 5’GCC CCA CTC GTT ATC ATA CC 3’

Appendix B: List of Strains Used

Strain	Genotype
WU27	G186AS background auxotrophic for uracil (Δura5-11) from Washington University
USM14Δ*95*	WU27 background Δ*MS95*
USM11	USM14Δ*95* background contains pEMS01 (*MS95* ORF)
USM12	USM14Δ*95* background contains pEMS01 (*MS95* ORF)
USM13	USM14Δ*95* background contains pEMS01 (*MS95* ORF)